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Differential expression of the eicosanoid pathway in patients with localized or mucosal cutaneous leishmaniasis

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Footnote page

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Abstract

Unfettered inflammation is thought to play critical role in the development of different clinical forms of tegumentary leishmaniasis. Eicosanoids are potent mediators of inflammation and tightly associated with modulation of immune responses. In this cross-sectional exploratory study, we addressed whether targets from the eicosanoid biosynthetic pathway assessed through multiplexed expression assays in lesion biopsies as well as in plasma could highlight a distinct biosignature in patients with mucocutaneous leishmaniasis (MCL) or localized cutaneous leishmaniasis (LCL). Differences in immunopathogenesis between MCL from LCL may result from an imbalance between prostaglandins and leukotrienes, which may serve as targets for future host-directed therapies.

Background

Tegumentary leishmaniasis is a vector-borne-disease caused by *Leishmania* parasites and exhibits a wide spectrum of clinical presentations. The most common clinical form of the disease caused by *L. braziliensis* parasites is the localized cutaneous leishmaniasis (LCL), characterized by ulcerated dermal lesions, which usually heal spontaneously [1]. A More severe form of this disease, mucocutaneous (MCL), is observed in 3% of individuals with LCL [2]. MCL patients usually present with severe and progressive destruction of nasopharyngeal and/or laryngeal structures [2]. MCL lesions exhibit an intense inflammation and tissue damage and paradoxically very few parasites. Necrosis of mucosal tissue is associated with a strong T-cell mediated response, reflected by an exacerbated delayed-type hypersensitivity (DTH) reaction to *Leishmania* antigens [3]. Possible mechanisms linked to increased disease severity in MCL are still unknown, but lack of immune modulation leading to uncontrolled inflammation seems to be critically involved [3].

Eicosanoids have been described to regulate key aspects of the host immune responses during *Leishmania* infection [4]. Prostaglandin E_2 (PGE_2) has been shown to benefit parasite survival whereas increased Leukotriene B_4 (LTB_4) production leads to enhanced intracellular parasite killing by infected host cells [5, 6]. These findings suggest that the balance between prostanoids and leukotrienes may directly affect the capacity of the host to control *Leishmania* infection. However, whether this dichotomy in the expression of eicosanoids is relevant in the context of MCL remains unknown.

Here, we perform a cross-sectional exploratory study in patients with MCL and LCL from an endemic area in Brazil assessing circulating levels as well as *in situ* RNA expression of mediators from the eicosanoid pathway. We identify a distinct biosignature

of MCL hallmarked by decreased expression of enzymes and receptors of prostaglandins and lipoxins compared with LCL. Moreover, plasma levels for PGE_2 and LTB_4 indicated that MCL patients are prone to skew the eicosanoid balance towards leukotrienes, whereas LCL individuals exhibit an enriched prostanoid signature. These distinct expression profiles have potential implications for the understanding of tegumentary leishmaniasis pathogenesis, which can lead to development of new host-directed therapies targeting the eicosanoid pathway.

Patients and methods

This study was approved by the institutional review board from Centro de Pesquisas Gonçalo Moniz, FIOCRUZ (number 136/2007). All clinical investigations were conducted according to the Declaration of Helsinki. Written informed consent was obtained from all participants or legal guardians and all data analyzed were anonymized.

The present study assessed age and gender- matched MCL (n=13, 1.4 male/female ratio, aged 59 ±17 years old) and LCL patients (n=29, 1.9 male/female ratio, aged 34 ±15 years old) (MCL vs. LCL age, P=0.894; gender distribution, P=0.921) recruited at our reference clinic in Jiquiriçá, Brazil. Individuals included in the present study were required to have no previous diagnosis of tegumentary leishmaniasis and to be treatment naïve. For plasma analyses, we included samples from 43 healthy endemic controls (age and gender matched with LCL and MCL) exhibiting negative DTH responses. LCL and MCL diagnosis was confirmed by the presence of ulcerated skin lesion or granulomatous mucosal lesion, respectively, in addition to at least one of the following positive tests: anti-*Leishmania* DTH, anti-*Leishmania* antibody titles or detection of *Leishmania* parasites in biopsy tissues by either immunohistochemistry or

qualitative polymerase chain reaction (PCR) assays. LCL patients exhibited a single or few ulcerated lesions for up to 2 months, while MCL patients had symptoms for a prolonged period (mean time of disease 10 ± 14 years) with mucosal lesions involving the nasal cavity (100%), pharynx (35%) and/or larynx (11%). Tissue samples from which we had high quality mRNA were obtained from a subset of 4 MCL and 7 LCL patients. These patients were similar to their respective groups with regard to age and gender (data not shown). Nasal mucosal samples were obtained from turbinoplasty nasal surgery and performed under local anesthesia. All tissue specimens were obtained before treatment.

Total RNA was extracted from cryopreserved lesion biopsies using Trizol reagent (Invitrogen, Carlsbad, CA), with an additional purification step using RNeasy columns (Qiagen, Venlo, Netherlands) as previously described [7]. nCounter (NanoString Technologies, Seattle, WA) analysis was performed at the VIB MicroArray Facility (Leuven, Belgium) based on direct molecular bar coding of target RNA transcripts and digital detection [7]. The chosen targets were: *PGES (PGE Synthase)*, *PGDS* (PGD Synthase), *PGD2R* (PGD 2 receptor), *PTGFR* (PGF receptor), *PTGS1* (COX-1), *PTGS2* (COX-2), *PLAS2G4A* (Phospholipase S2G4A), *PLA2G6* (Phospholipase 2G6), *LXA4R* (Lipoxin A4 receptor), *PTGER1* (E-prostanoid receptor 1), *PTGR2* (E-prostanoid receptor 2), *PTGER3 (E-prostanoid receptor 3)*, *PTGER4* (E-prostanoid receptor 4), *ALOX15* (Arachidonate 15-lipoxygenase), *ALOX12* Arachidonate 12-lipoxygenase, *ALOX5* (Arachidonate 5-lipoxygenase). To account for differences in leukocyte infiltration between patient lesions, data were normalized for *CD45*, which encodes the pan-leukocyte marker CD45, detectable at the femtomolar range as previously reported [7].

Concentrations of PGE_2, PGD_2 and $PGF_{2\alpha}$, LTB_4 and resolvin D1 (RvD1) were measured in cryopreserved EDTA-treated plasma samples from all patients using an enzyme-linked immunoassay (Cayman Chemical, Ann Harbor, MI).

Median values with interquartile ranges (IQR) were used as measures of central tendency. For expression assays, Mann-Whitney test was used to compare the variables. Plasma values were compared using the Kruskal-Wallis test with Dunn's multiple comparisons ad hoc test. Unsupervised two-way hierarchical cluster analyses (Ward's method) with bootstrap were employed to test whether MCL and LCL patients can be grouped separately based of simultaneous assessment of plasma eicosanoids. Two models of Principal Component Analysis (PCA) were employed to test the contribution of PGE_2 levels to the power of the combined assessment of several eicosanoids to distinguish MCL from LCL cases. A P-value < 0.05 was considered statistically significant.

Results and Discussion

To characterize the eicosanoid signaling pathways expressed *in situ* during MCL and LCL, we performed a comprehensive analysis of targeted RNA transcripts isolated from mucosal vs. skin biopsies. The target transcripts were represented within the context of an eicosanoid signaling pathway using Ingenuity Pathway Analysis (Figure 1A). Remarkably, MCL patients exhibited a substantial down modulation in several genes from the prostaglandin pathway compared with those with LCL (Figure 1B). Among all the genes examined, we found that *PGES*, *PTGER3*, *PGDS*, *PTGFR*, *PTGS1* and *ALOX5* expression were significantly lower whereas LXA4R expression was higher in MCL cases than in individuals with LCL (Figure 1C).

Interestingly, differences found in expression of constitutively expressed targets, such as *PTGS1* gene/COX-1, and for the inducible isoforms, such as *PTGS2* gene/COX-2, indicate that LCL and MCL activate distinct prostaglandins synthase pathways. PGE_2 acts through four distinct G protein-coupled receptors, E-prostanoid (EP) 1, EP2, EP3 and EP4 [8]. Once PGE_2 binds to different receptors, it can activate different signaling pathways inducing multiple, and sometime paradoxical, effector functions. Notably, it has been reported that *Leishmania major* infection up-regulates EP1 and EP3 expression while down-regulating EP2 and EP4 *in vitro* [9]. Our analyses suggested that differential expression of EP3 might be an important parameter related to the pathogenesis of MCL and LCL. Our exploratory findings warrant the design of additional studies that assess the role of EP receptors signaling in leishmaniasis.

We next tested whether the differences in gene expression observed *in situ* could be reflected in a distinct profile of plasma concentrations of in MCL and LCL patients. By quantifying plasma levels of PGE_2, PGD_2 and $PGF_{2\alpha}$, as well as LTB_4 and RvD1, we found that LCL patients exhibited a distinct expression profile compared with MCL (Figure 2A). We observed that PGE_2 levels were significantly higher in LCL cases compared with individuals with MCL (Figure 2B). Whether the augmented circulating levels of these prostanoids are directly related to the increased COX1/*PTGS1* expression in skin lesions observed in LCL patients deserves future clarification. Importantly, compared with values detected in healthy endemic controls, PGD_2, $PGF_{2\alpha}$, LTB_4 and RvD1 were significantly lower in LCL patients (Figure 2B). Conversely, concentrations of all the eicosanoids, except for $PGF_{2\alpha}$, were undistinguishable between MCL and controls (Figure 2B). Noteworthy, levels of LTB_4 were >1log higher in MCL patients than in LCL cases (Figure 2B). Leukotrienes are highly bioactive and minor differences

in plasma measurements could reflect major differences in inflammation, as observed in other disease models [10]. These results indicate that a down modulatory effect may be more relevant in LCL than in MCL compared to healthy donors. Thus, systemic mediators observed in LCL and MCL may be useful as biomarkers of active disease.

Together, these observations led us to hypothesize that a balance in the circulating levels of lipid mediators is associated with differential inflammatory status observed in MCL or LCL. In this scenario, prostaglandins derived from cyclooxygenases would prevail over lipoxygenase-derived products in LCL patients compared with those with MCL. To test this hypothesis, we performed an unsupervised hierarchical cluster analysis in which plasma values of all the eicosanoids measures from MCL and LCL cases were inputted. We confirmed that simultaneous assessment of key prostaglandins, LTB_4 and RvD1, could successfully segregate the different clinical groups evaluated (Figure 2C).

RvD1, an important specialized pro-resolving mediator, is endogenously generated during the spontaneous resolution phase in many models of acute and chronic inflammation diseases [11]. Counterintuitively at a first glance, although our data reveals that there is no statistically significant difference for RvD1 plasma levels between LCL and MCL patients, we noticed a trend of RvD1 median levels to be approximately 1.2 log decreased in LCL compared with MCL (Figure 2B). Whether RvD1 participates in the control and resolution of inflammation or promotion of parasite survival in tegumentary leishmaniasis patients needs to be further investigated.

Notably, to our knowledge, this is the first study to demonstrate increased levels of prostanoids in plasma of tegumentary leishmaniasis patients. Interestingly, we found that $PGF_{2\alpha}$ was in general reduced in tegumentary leishmaniasis patients compared to controls (Figure 2B). Recent studies from our group reported that $PGF_{2\alpha}$ is uniquely

involved in the cellular metabolism of *Leishmania* species and its immune evasion capacity in murine models [12, 13]. Modulation of the $PGF_{2\alpha}$ production could be a potential mechanism by which the host restricts a key mediator for promotion of parasite growth.

Strikingly, additional hierarchical clustering analyses confirmed that the groups of MCL and LCL patients could be better separated when data on only two eicosanoids, PGE_2 and LTB_4, were considered (Figure 2C). The balance between these eicosanoids has been described to determine clinical outcomes in other diseases [10]. Two models of PCA supported the idea that differential expression of PGE_2 in plasma is probably the most important parameter leading to distinction between MCL and LCL patients (Figure 2D). Importantly, values of PGE_2/LTB_4 ratio were >8-fold higher in LCL patients than in MCL cases (median and IQR: 5.3, 3.5-6.4 vs. 0.6, 0.1-0.8; $P<0.0001$). Thus, circulating levels of PGE_2 and LTB_4 could be tested as potential biomarkers of mucosal involvement in tegumentary leishmaniasis. Considering that MCL cases exhibit longer periods with disease prior to diagnosis and that this disease form may progress from localized lesions, it is possible that our results may be affected by illness duration. Prospective studies focused on early detection of MCL may help clarifying this issue. In addition, differences in the expression profile of these biomarkers may reflect distinctions in the infiltration of leukocytes in the lesions. Although MCL and LCL exhibit in general cellular infiltrates enriched for mononuclear cells, we have previously shown an important role for neutrophils contributing to inflammation in MCL [14]. Cellular analyses employing flow cytometry could be performed to extensively phenotype cellular subsets recruited to tegumentary lesions, thus elucidating associations between the leukocyte infiltrate and the differential eicosanoid expression described here. A limitation of the present study

was lack of assess to skin/mucosal biopsies from healthy endemic controls for comparison with those from leishmaniasis patients. In addition, we could not test correlations between eicosanoid expression and parasite burden in the lesions, because the parasite quantification was very low and the sensitivity of the histological technique was insufficient to provide reliable quantitative values in such small sample set of individuals from whom we had in situ data. Regardless, our data indicate that, eicosanoids pathways, and PGE_2 in particular, may be explored as novel targets for therapeutic interventions of tegumentary leishmaniasis.

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Figure legends

Figure 1. Differential expression of selected genes of eicosanoid pathways in skin or mucosal lesions from patients with tegumentary leishmaniasis. Total RNA was extracted from lesion biopsy specimens for patients with LCL (n = 7) and those with MCL (n = 4). Indicated messenger RNA transcripts of host-specific cellular genes were quantified by nCounter (Nanostring), including the pan-leukocyte gene CD45, for normalization of immune infiltration into tissues. (A) The targeted genes were represented within the context of an eicosanoid signaling pathway using Ingenuity Pathway Analysis. Red and green colors infer higher or lower gene expression in MCL relative to LCL patients, respectively. (B) A representative profile of geometric mean values (log10-transformed) for indicated genes is displayed for each clinical group. (C) Scatter plots of gene expression relative to CD45 are shown. Lines represent median values and interquartile ranges. Data were compared using the Mann-Whitney test. *P<0.05

Figure 2. Plasma concentrations of eicosanoids in patients with localized or mucosal cutaneous leishmaniasis.

(A) Plasma levels of COX-2 derived prostanoids PGE_2, PGD_2 and $PGF_{2\alpha}$ and well as 5-LO derived lipid mediators LTB_4 and RvD1 were compared between patients with localized (LCL; n=29) or mucosal (MCL; n=13) leishmaniasis as well as healthy endemic controls (EC; n=43). Data were compared using the Kruskal-Wallis test with Dunn's multiple comparisons ad hoc test (*P<0.05, **P<0.01, ***P<0.001). Lines represent median values and interquartile ranges. **(B)** Univariate analyzes with scatter plots of the comparisons are shown. **(C)** A hierarchical clustering analysis (Ward's method) was employed to test whether the overall expression profile of all the lipid mediators (upper panel) or just PGE_2 and LTB_4 (lower panel) in plasma could distinguish MCL from LCL cases. **(D)** Two principal component analysis (PCA) models were utilized to examine the contribution of PGE_2 in explaining the differences observed between the LCL and MCL study groups. PC, principal component.